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The full-length E1^E4 protein of human papillomavirus type 18 modulates differentiation-dependent viral DNA amplification and late gene expression

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Abstract

Activation of the productive phase of the human papillomavirus (HPV) life cycle in differentiated keratinocytes is coincident with high-level expression of E1^E4 protein. To determine the role of E1^E4 in the HPV replication cycle, we constructed HPV18 mutant genomes in which expression of the full-length E1^E4 protein was abrogated. Undifferentiated keratinocytes containing mutant genomes showed enhanced proliferation when compared to cells containing wildtype genomes, but there were no differences in maintenance of viral episomes. Following differentiation, cells with mutant genomes exhibited reduced levels of viral DNA amplification and late gene expression, compared to wildtype genome-containing cells. This indicates that HPV18 E1^E4 plays an important role in regulating HPV late functions, and it may also function in the early phase of the replication cycle. Our finding that full-length HPV18 E1^E4 protein plays a significant role in promoting viral genome amplification concurs with a similar report with HPV31, but is in contrast to an HPV11 study where viral DNA amplification was not dependent on full-length E1^E4 expression, and to HPV16 where only C-terminal truncations in E1^E4 abrogated vegetative genome replication. This suggests that type-specific differences exist between various E1^E4 proteins.

Keywords: Human papillomavirus; HPV; HPV18; HPV life cycle; HPV replication; E1^E4; E4 protein; Epithelial differentiation; Cell proliferation

Introduction

Infection of epithelial tissues by human papillomaviruses (HPV) produces a range of clinical lesions including common warts, genital warts and cancers of the oral cavity and anogenital tract. Of the greater than 200 different HPVs that have been identified, HPV types 16 (HPV16) and 18 (HPV18) are the most common of the virus types detected in carcinomas of the cervix, head and neck (Bosch et al., 1995; Castellsague et al., 2006; Kreimer et al., 2005). HPVs infect cells in the basal layer of squamous epithelia. In these undifferentiated cells the double-stranded DNA viral genome is established as an extrachromosomal plasmid and maintained at a low copy number. Upon cellular differentiation, amplification of the HPV genome occurs, followed by expression of the capsid proteins L1 and L2, and eventual progeny virion synthesis (Hebner and Laimins, 2006). Because HPV DNA synthesis requires the host cell replication machinery, the virus stimulates the differentiating keratinocytes to re-enter S-phase in order to express essential cell replication factors and enable viral DNA amplification (Hebner and Laimins, 2006).

Initiation of the productive phase of the HPV life cycle is accompanied by induction of E4 expression and high levels of this HPV protein persist throughout the virus-producing phase (Peh et al., 2002). The E4 protein is translated from spliced E1^E4 transcripts and contains the first five amino acids from the E1 protein fused to the E4 coding sequence (Nasseri et al., 1987). Transcripts encoding the E1^E4 open reading frame (ORF) are up-regulated upon cellular differentiation, but E1^E4-containing transcripts are also present in undifferentiated cells

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(Hummel et al., 1992). However, in undifferentiated cells, E1^E4 is the third ORF from the 5′ end of most early transcripts and is translated only at low levels. The function of E4 in the HPV life cycle is not known, but over-expression studies of E4 in epithelial cells have revealed several potential physiologically relevant functions. High-levels of E1^E4 lead to disruption of sub-nuclear structures known as nuclear dot 10 (ND10 domains) and this maybe advantageous for successful virus replication (Roberts et al., 2003). ND10 domains are sites intimately involved in the replication of many DNA viruses (Everett, 2006), and the disruption of ND10 domains by E1^E4 is seen in cells competent for HPV1 replication (Roberts et al., 2003). E1^E4 has also been hypothesized to facilitate the release of newly-synthesized virions from the upper cells of lesions, by inducing a weakening of the cornified cell envelope (Bryan and Brown, 2000) and disturbing the intermediate filament cytoskeleton (Doorbar et al., 1991; Roberts et al., 1993). In addition, E1^E4 has been shown to associate with mitochondria and induce apoptosis in a manner that may facilitate the late stage of the virus life cycle (Raj et al., 2004). More recent studies demonstrated that over-expression of E4 interferes with cell proliferation and causes cells to arrest in G2. This property is conserved between

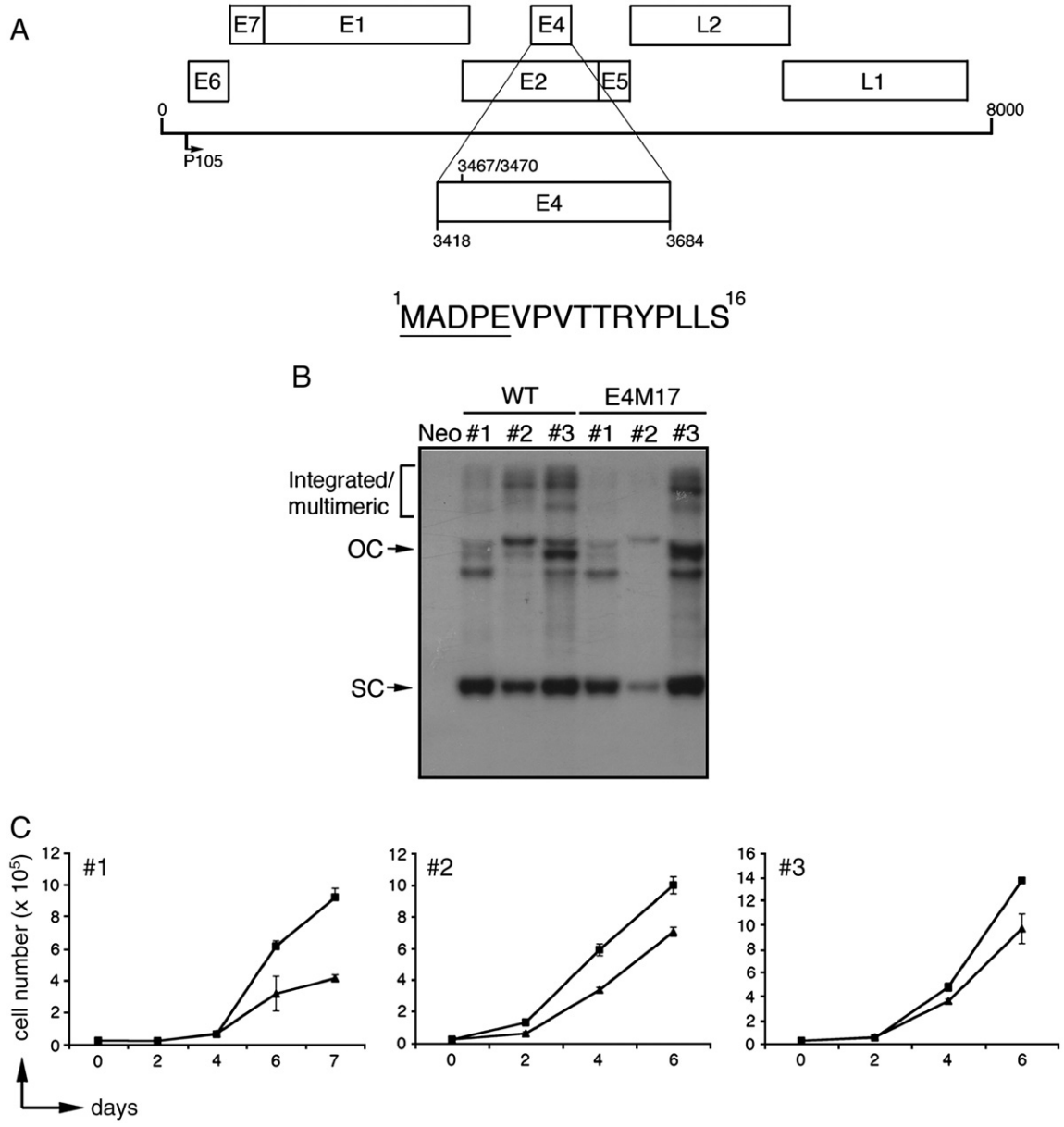

Fig. 1. HPV18 genome-containing foreskin keratinocytes unable to express full-length E1^E4 show increased cell proliferation compared to wildtype cells, but are not impaired in stable viral DNA replication. (A) Two mutations introduced at nucleotides 3467 and 3470 of the HPV18 genome insert two translation termination codons in the E4 ORF. The nucleotide changes are silent in the overlapping E2 coding sequence. The mutant HPV18 genome is predicted to express a truncated E1^E4 protein of 16 amino acid (the E1 exon is underlined). (B) Southern analysis of equal amounts of total DNA prepared from foreskin keratinocytes, transfected with a drug selection plasmid alone (Neo), HPV18 wildtype (WT) or mutant E4 (E4M17) genomes, grown as monolayer cultures to similar passage number, and probed with a linear form of the HPV18 genome. HPV18 episome forms migrate as supercoils (SC) and open-circles (OC). (C) Cell growth profiles, performed in duplicate, of three donor keratinocytes (#1, #2, and #3), harbouring HPV18 wildtype (▲) or mutant E4 (■) genomes.

the E4 proteins of HPV types with diverse tissue tropism and pathogenicity (Davy et al., 2002; Knight et al., 2004; Nakahara et al., 2002). Since inhibition of the G2-to-M transition can also stimulate cellular DNA synthesis (Knight et al., 2004; Nakahara et al., 2002), this E4 function may facilitate viral genome replication in suprabasal keratinocytes.

A genetic analysis of the E4 ORF in complete cottontail rabbit papillomavirus (CRPV) genomes indicated that expression of a full-length E1^E4 protein is crucial for activation of viral late functions. Whilst papilloma formation was unaffected by loss of E4 expression, both amplification of the viral genome and capsid expression were inhibited (Peh et al., 2004). A similar requirement for full-length E1^E4 protein expression was necessary for efficient induction of HPV31 late functions in differentiated keratinocytes, and loss of E1^E4 correlated with a failure of suprabasal cells to re-enter the cell cycle (Wilson et al., 2005). In contrast, studies with HPV11 failed to demonstrate any role for E1^E4 in the productive phase of the viral life cycle (Fang et al., 2006). Similarly, studies of HPV16 indicated that truncations of E4 at the N terminus had little inhibitory effect on the activation of late functions, in contrast to truncations of the C-terminus which had a significant effect (Nakahara et al., 2005). Thus, different activities have been reported for E1^E4 proteins of various high and low risk HPV types. To ascertain whether the productive virus life cycle of other high-risk HPV types is dependent on full-length E4 functions, we have investigated the effects of the loss of E1^E4 function on the HPV18 life cycle.

Results

Loss of full-length E1^E4 expression alters cell proliferation of undifferentiated keratinocytes, but does not affect stable maintenance of the HPV18 genome

In order to investigate the role of the full-length E1^E4 protein in the life cycle of HPV18, we constructed mutant genomes that contained two point mutations, T^{3467}→A and T^{3470}→G, that are silent in the E2 ORF, but which introduce two translation termination codons at positions 17 and 18 of the E1^E4 coding sequence. This results in the synthesis of a truncated E1^E4 protein of 16 amino acids (Fig. 1A). Wildtype and mutant genomes were each co-transfected with a neomycin resistance plasmid into primary human foreskin keratinocytes (HFKs) obtained from individual foreskin donors, and after a brief selection period the isolated clones were pooled. To ensure that any effect upon the HPV18 life cycle is not specific to one HFK isolate, three different foreskin donors were used to generate cell lines containing HPV18 wildtype (WT#1, #2, and #3) or mutant E4 (E4M17#1, #2, and #3) genomes.

The contribution of E1^E4 function to the early phase of HPV18 genome replication was evaluated by Southern analysis of total genomic DNA isolated from undifferentiated monolayer cultures of HPV18 containing HFKs. The DNA samples were digested with *Dpn*I to remove residual input bacterial DNA, and *Bgl*II that has no recognition sites in the HPV18 genome. Southern analysis using an HPV18 genomic probe revealed the presence of HPV18 episomes, migrating as supercoiled (SC) and open circle (OC) forms, in both wildtype and mutant E4 lines of all three donor isolates (Fig. 1B). In one foreskin donor (#2), the level of mutant E4 episomes was moderately lower than that of wildtype genomes. However, this effect on episome maintenance was not observed in the two other HFKs (Fig. 1B), even upon extended passage (data not shown). We conclude that full-length E1^E4 is not required for establishment or stable replication of HPV18 genomes in normal keratinocytes.

Since studies using heterologous expression systems indicated that HPV E4 protein expression is associated with suppression of keratinocyte growth and induction of a G2 arrest of the cell cycle (Davy et al., 2002; Knight et al., 2004; Nakahara et al., 2002), we next compared the growth rates, and the cell cycle profile, of the established HPV18 wildtype- and E4 mutant-containing cell lines grown in monolayer culture. At various time points, keratinocytes were harvested and viable cells counted after removal of feeder fibroblasts (Fig. 1C). In all sets of transfected HFKs using different donor backgrounds, the growth rate of cells containing the mutant E4 genomes was significantly greater than those containing wildtype genomes (Fig. 1C). This suggests that HPV18 E1^E4 functions are relevant in undifferentiated cells prior to the onset of viral late functions. The significance of a possible role for E1^E4 in the regulation of proliferation of cells in the early phase of the HPV life cycle is not clear, but our observations are in-keeping with the observed increase in papilloma formation by CRPV genomes unable to express E4 (Peh et al., 2004). Cell cycle analysis did not identify a difference between the profiles of cells containing wildtype or mutant E4 HPV18 genomes (data not shown).

HPV18 genome amplification and late gene transcription are reduced in keratinocytes containing mutant E4 genomes following differentiation by suspension in methylcellulose

To ascertain whether E1^E4 has a role in the productive phase of the HPV18 replication cycle, viral late functions were induced by suspension of the keratinocyte cell lines in 1.5% methylcellulose. Total genomic DNA, and RNA, was prepared from cells prior to suspension in methylcellulose (0 h), and after 24 and 48 h in suspension. Induction of differentiation-dependent HPV18 genome amplification and late gene expression were examined by Southern and Northern analyses respectively. Fig. 2A shows a typical Southern blot of cells containing wildtype or mutant E4 HPV18 genomes. Whilst a significant level of differentiation-dependent DNA amplification occurred upon suspension in wildtype genome-containing cells, there was little amplification of the mutant E4 genomes, even after 48 h in methylcellulose. Failure of keratinocytes to support efficient amplification of the mutant genome was consistently observed in transfected cell lines from all three donor HFKs (Fig. 2B). Northern blot analysis of total cellular RNAs isolated form keratinocytes grown in monolayer culture (0 h), demonstrated no differences in the levels of early viral transcripts between cells containing wildtype or mutant E4 genomes (Fig. 2C).

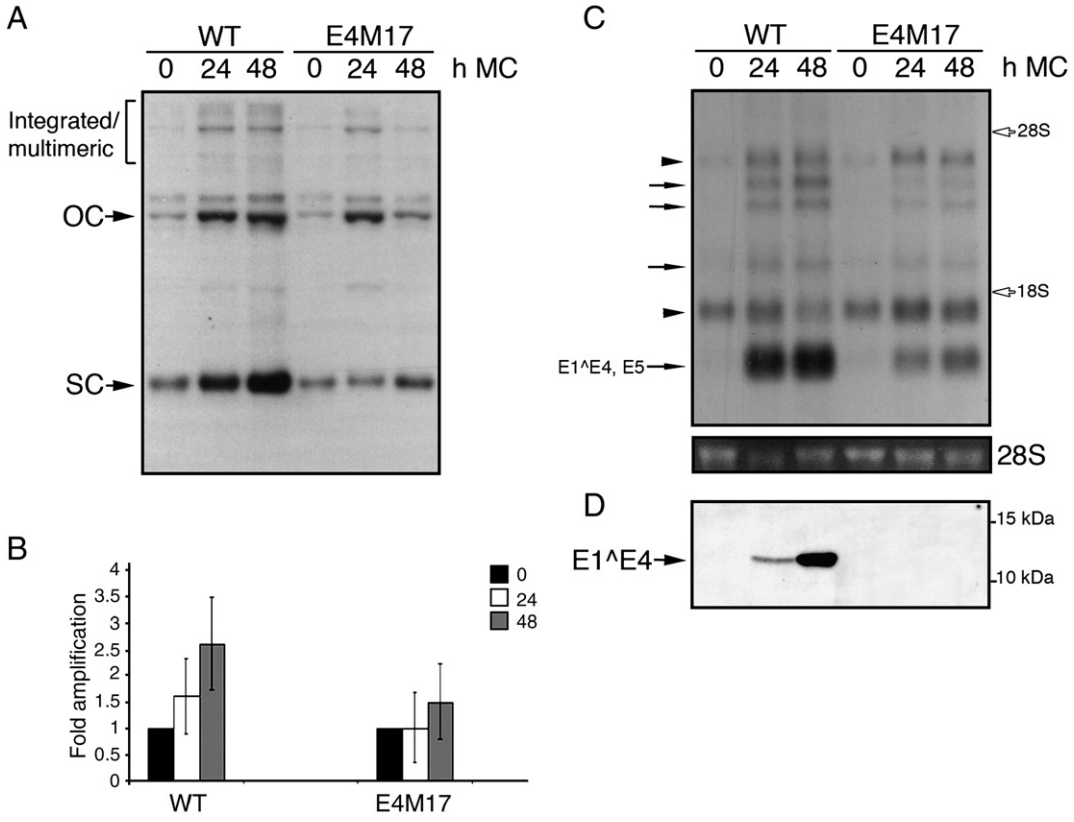

Fig. 2. Loss of full-length E1^E4 expression impairs differentiation-dependent HPV18 DNA amplification and activation of late gene expression. (A) Southern analysis of equal amounts of total DNA extracted from HFKs transfected with wildtype (WT) or mutant E4 (E4M17) genomes and suspended in methylcellulose (MC) for various times. HPV18 episome forms migrate as supercoils (SC) and open-circles (OC). (B) Bar graph showing amplification of WT and E4M17 genomes in a different donor keratinocyte population. Data was derived from phosphoimaging analysis of six experiments using three matched sets of cells with WT and E4M17 genomes. (C) Northern analysis of equal amounts of total RNA extracted from HPV18 genome-containing HFKs following suspension in MC. Arrowheads mark the positions of early transcripts, and arrows indicate late transcripts. Migration of 28S and 18S RNAs is as indicated and the level of 28S RNA is a loading control. A linear form of the HPV18 genome was used as a probe for HPV18 DNA and RNA. (D) Detection of E1^E4 protein expression by Western blot analysis of total protein lysates.

Upon differentiation, induction of HPV18 late gene expression occurred efficiently in keratinocytes transfected with wildtype genomes, with significant production of the late transcripts encoding E1^E4, E5 (Fig. 2C). However, in comparison to the keratinocytes containing the wildtype genomes, the induction of these late transcripts was severely inhibited in cells containing the mutant E4 genome, with only a low level of the transcripts accumulating in cells following suspension in methylcellulose for 48 h (Fig. 2C). Western blot analysis of E1^E4 expression was performed with a cross-reactive monoclonal antibody (1D11) that is specific to the N-terminus of E1^E4 proteins (Roberts et al., 2003). This analysis showed that induction of wildtype HPV18 genome amplification correlated with high-level expression of E1^E4 protein (Fig. 2D). As expected, we were unable to detect an E4 polypeptide in mutant E4 genome transfected cells (Fig. 2D). However, we cannot rule out the possibility that the truncated 16 amino acid N-terminal E1^E4 polypeptide that would be produced in these cells (Fig. 1A) is stable, and furthermore, that it is functional. Overall these observations indicate that the full-length HPV18 E1^E4 protein is required for the differentiation-dependent amplification of viral genomes and induction of late gene expression.

Suprabasal DNA synthesis is not inhibited in organotypic raft cultures of keratinocytes containing mutant E4 genomes

Previous studies with HPV31 demonstrated a reduction in the number of S-phase nuclei retained in the suprabasal cell layers of organotypic raft cultures of cells containing genomes unable to express E1^E4 (Wilson et al., 2005). This suggested that HPV31 E1^E4 might support differentiation-dependent viral late functions by contributing to the activation, or the maintenance of an S phase state in suprabasal cells (Wilson et al., 2005). To examine whether S-phase re-entry of suprabasal cells is similarly affected by mutant HPV18 E4 genomes, organotypic rafts cultures of the HPV18 transfected cells were prepared. To identify nuclei undergoing DNA synthesis, rafts were incubated with the thymidine analogue bromodeoxyuridine (BrdU), prior to harvesting 12 h later, and sections stained with a BrdU monoclonal antibody. While DNA synthesis was largely restricted to cells of the basal cell layer in raft cultures of untransfected HFKs, in wildtype and mutant E4 rafts, BrdU-positive nuclei were detected in both basal and suprabasal epithelial compartments (Fig. 3A). Moreover, there was no difference found in the number of BrdU-positive suprabasal nuclei present in raft cultures of cells containing wildtype or mutant E4 genomes (Fig. 3A and data not

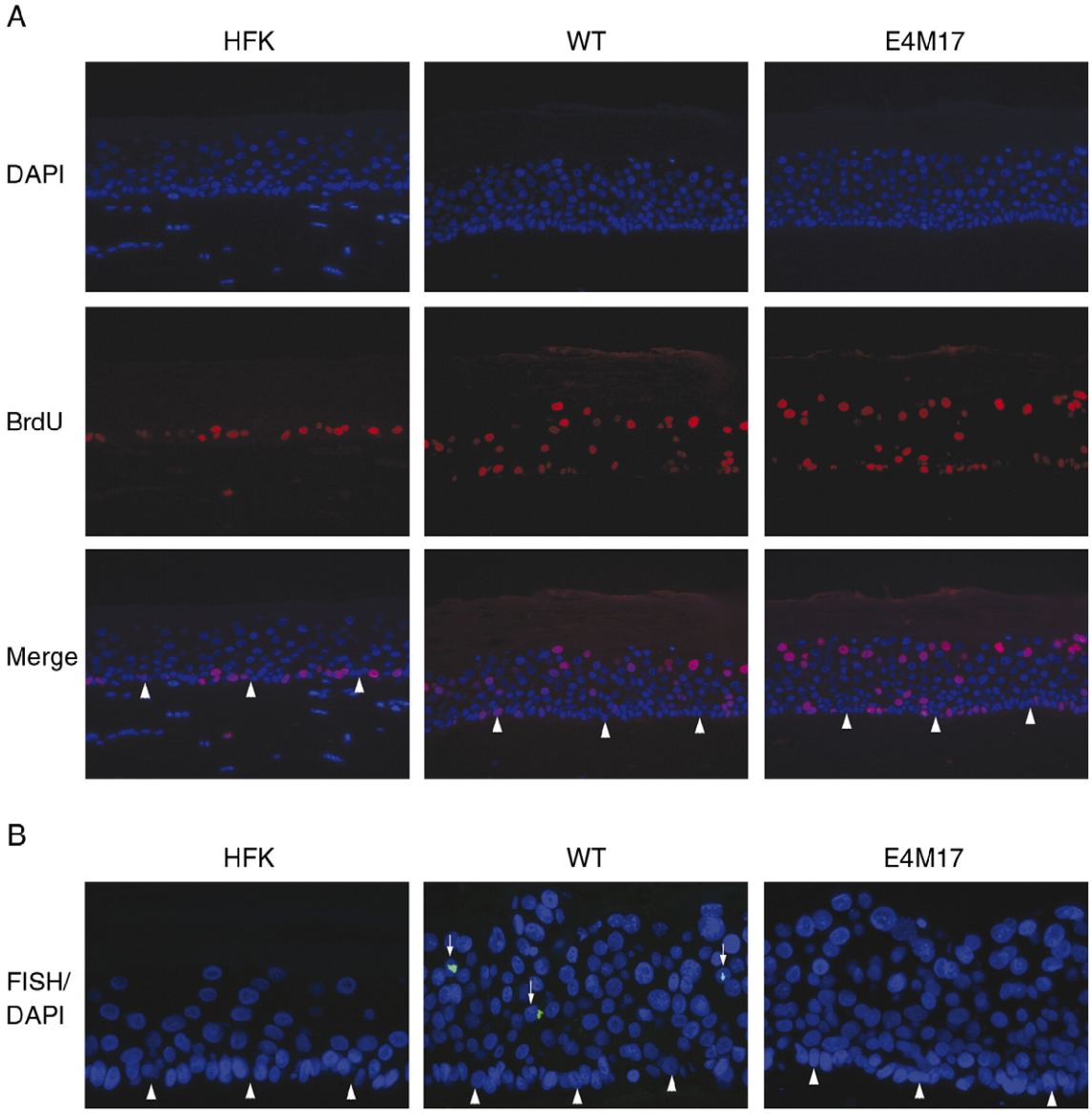

Fig. 3. Suprabasal DNA synthesis is not compromised by a loss of full-length E1^E4 expression. (A) Organotypic rafts cultures of keratinocytes transfected with wildtype (WT), mutant E4 (E4M17) genomes or untransfected cells (HFK), were incubated with BrdU to identify nuclei positive for DNA synthesis. Immunofluorescence was performed with an anti-BrdU antibody (red) and all nuclei were visualized using DAPI-staining (blue). Arrowheads indicate the basal layer. (B) Nuclei positive for HPV18 genome amplification were identified by FISH analysis (green, arrows). Arrowheads indicate the basal layer.

shown). This indicates that loss of full-length E1^E4 expression does not compromise the ability of HPV18-containing keratinocytes to undergo S phase re-entry upon differentiation. In order to detect nuclei positive for viral DNA amplification, fluorescence in situ hybridization (FISH) analysis was performed using HPV18 genomic DNA as a probe. In rafts prepared from wildtype HPV18 genome-containing cells, suprabasal nuclei positive for viral DNA amplification were detected, but not in raft cultures of untransfected or mutant E4 cells (Fig. 3B), confirming our finding obtained from growth of cells in methylcellulose (Fig. 2A).

To confirm loss of E4 expression in the mutant E4 raft cultures, immunocytochemistry using rabbit antibodies prepared against a HPV18 E1^E4-GST fusion protein was performed on raft sections. Expression of E4 was detected in suprabasal cells of rafts formed by keratinocytes containing the wildtype genomes, but not untransfected cells, or those transfected with mutant E4 genomes (Fig. 4). Interestingly, the pattern of E4-staining in the wildtype raft cultures changed as the cells differentiated, appearing as a cytoplasmic fibrous distribution in nucleated cells, but becoming more punctate in the cytoplasm of superficial cells (Fig. 4).

Discussion

In this study, we have shown that normal foreskin keratinocytes containing HPV18 genomes unable to express a full-length E1^E4 protein, fail to support efficient viral genome amplification upon differentiation. Loss of E1^E4 expression is also associated with an inhibition of activation of viral late gene expression. We conclude that expression of full-length E1^E4 is

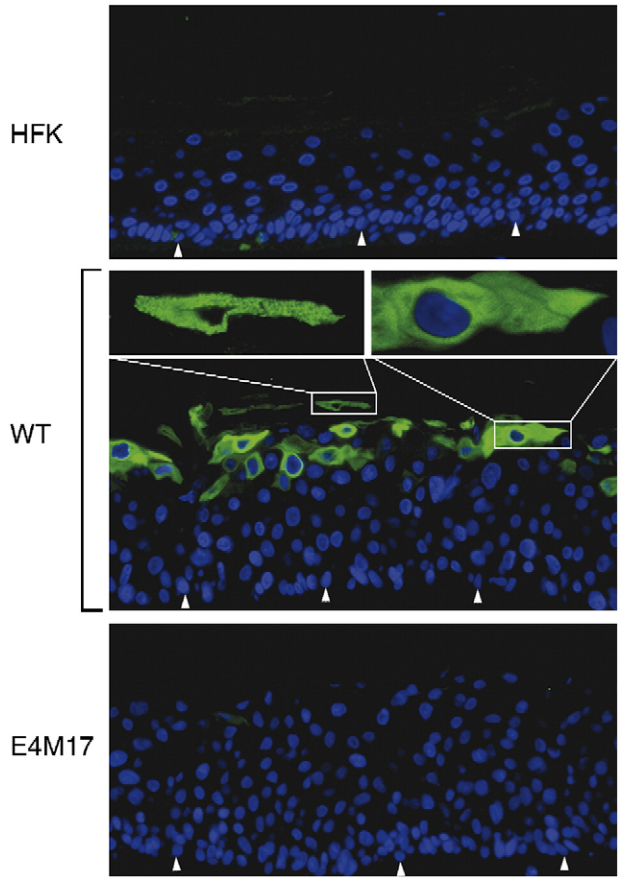

Fig. 4. HPV18 E1^E4 expression in organotypic raft cultures. E1^E4 protein accumulates in suprabasal cells of keratinocytes containing wildtype (WT), but not mutant E4 (E4M17) genomes, or untransfected keratinocytes (HFK). Note that as WT cells migrate upwards in the raft the distribution of E4 staining changes. Examples of nucleated cells with a cytoplasmic fibrous E4 staining (right hand panel) and a more superficial cell showing punctate E4 distribution (left hand panel) are shown at high magnification.

required for efficient execution of the productive phase of HPV18. Our findings are consistent with those of two previous studies, one of the life cycle of CRPV *in vivo* (Peh et al., 2004), and the other that examined the HPV31 replication cycle in normal foreskin keratinocytes induced to differentiate in tissue culture (Wilson et al., 2005). Both identified a critical role for E4 in papillomavirus genome amplification and late gene expression. However, it has recently been reported that expression of a full-length E1^E4 protein was not necessary for efficient HPV11 genome amplification in organotypic raft culture (Fang et al., 2006). One explanation for a non-essential role of E1^E4 in HPV11 genome amplification would be a difference in E1^E4 function between HPV types that exhibit dissimilar pathogenicity and tropism; HPV11 infections are primarily of the external genitalia and the larynx, with lesions having only a low-risk of becoming malignant, whilst infections induced by HPV16, 18 and 31 are most common at the mucosal sites of the genitalia and oral epithelia, and have a high-risk of malignant conversion. Indeed, E6 and E7 proteins of the high-risk virus types mediate different biological functions to those of the low-risk viruses (Hebner and Laimins, 2006).

In the HPV11 study, the mutant genomes express a truncated E1^E4 protein that terminates at a similar position as those of HPV18 and 31. For HPV11, the translation stop codon was introduced immediately before a conserved N-terminal leucine motif (HPV11, MADDSALYEKYP), while in the HPV18 and 31 proteins termination occurs within the motif itself (HPV18, MADPEVPVTTRYPLLS; HPV31, MADPAAVTKYPLLG, the initial part of the leucine motif LLXLL is underlined). Since it is known that the N-terminus of E1^E4 plays an important role in several biological properties of E4 (Raj et al., 2004; Roberts et al., 1994, 2003), it is plausible that the truncated E1^E4 peptide of HPV11, but not of HPV18 or 31, is able to mediate functions required to support viral DNA amplification. An alternative explanation for the differences in E1^E4 function could be that different experimental systems were used to assess the role of E1^E4 in the virus life cycles. The HPV11 life cycle was examined in N-Tert cells, a human foreskin keratinocyte cell line immortalized by the catalytic subunit of human telomerase (Fang et al., 2006), while in our analysis we transfected HPV18 genomes into normal primary foreskin keratinocytes, the same target cell as that used in the HPV31 study (Wilson et al., 2005). It is possible that the immortalized keratinocyte cell line compensates in some manner for the lack of those E1^E4 functions that might be required for viral DNA amplification. It is interesting to note that loss of E4 function in the more divergent virus type CRPV correlates with life cycle defects similar to those observed with HPV18 and HPV31 mutant genomes (Peh et al., 2004). It could be argued that since the analysis of CRPV E4 function involved re-introduction of the mutant CRPV genomes into the skin of rabbits (i.e. into primary keratinocytes), this study is analogous to those of HPV18 and HPV31 that were also performed in primary cells, but not the HPV11 study in immortalized cells.

A more complicated phenotype was reported in studies on HPV16 E1^E4 which indicated that the differentiation-dependent amplification phase of HPV16 genome replication was not significantly affected following transfection into normal immortalized keratinocytes (NIKS) of mutant genomes that expresses a similarly truncated E1^E4 protein (MADPAAATKYPLLK, 16st15) (Nakahara et al., 2005). However, deletion of the C-terminal third of E4 led to inhibition of HPV16 genome amplification, while truncation mutations that resulted in smaller HPV16 E1^E4 proteins seemed to enhance amplification. The authors hypothesized that these smaller truncations of HPV16 E1^E4 represent gain-of-function mutations. This study also used immortalized keratinocytes and this may contribute to the differences observed. Alternatively, it could reflect important type-specific differences.

Our studies also demonstrated that while loss of E1^E4 expression inhibited efficient HPV18 genome amplification in differentiating keratinocytes, there was no corresponding reduction of suprabasal cellular DNA synthesis. This is in contrast to studies examining keratinocytes containing the equivalent E1^E4 mutants of HPV16 (16st15) and HPV31 (E4M9) genomes, which both showed a decrease in suprabasal DNA synthesis (Nakahara et al., 2005; Wilson et al., 2005). While this action of E1^E4 might be significant for HPV16 and

31, it appears not to be a required activity for HPV18. This further underscores that fact that differences exist in the modes of action of E1^E4 proteins from various HPV types.

In addition to effects in differentiated cells we also observed effects due to HPV18 E1^E4 in undifferentiated cells. HFKs transfected with the mutant HPV18 E4 genomes showed enhanced proliferation of undifferentiated cells when compared to the growth of cells containing the wildtype genomes. Although E1^E4 protein expression is most abundant in cells competent for viral DNA amplification and virion production, transcripts containing the spliced E1^E4 genes are produced during the virus non-productive phase (Stubenrauch and Laimins, 1999). Genetic studies have demonstrated that E1 is expressed in undifferentiated cells (Frattini et al., 1996) so it is likely that E1^E4 is also synthesized in these cells as it shares common N-terminal amino acids. It is therefore possible that E1^E4 proteins contribute to an early viral function that affects cell growth. The effect of HPV18 E1^E4 on cell proliferation does not appear to be connected to viral DNA replication in undifferentiated cells since there were no obvious differences between the levels of episome forms between the wildtype or E4 mutant cells. A role for E1^E4 in the early phase of the virus life cycle is also supported by the observation that HPV16 DNA replication is inhibited in undifferentiated NIKS cells transfected with genomes carrying mutations within the N-terminal leucine-rich motif of E4 (Nakahara et al., 2005).

Overall our studies have demonstrated that E1^E4 from HPV18 plays an important role in regulating late viral functions. In addition, they demonstrate that type-specific differences exist between various E1^E4 proteins which is consistent with the high degree of sequence variability of this viral protein among the different HPV types.

Materials and methods

Construction of mutant HPV18 genomes

A pGEMII plasmid containing the complete HPV18 genome inserted at the *Eco*RI restriction site at nucleotide 2440 (pGEMII18-WT, a gift from Frank Stubenrauch, University of Tuebingen) was used as a template for site-directed mutagenesis (QuikChange, Stratagene) of the E4 ORF. Two nucleotide substitutions (T^{3467}→A and T^{3470}→G) were inserted into the E4 ORF using the primer set, 5′-CGG TAT CCG CTA CTC AGC TAG TGA AAC AGC TAC AGC ACA CCC-3′ (forward primer) and 5′-TGT GCT GTA GCT GTT TCA CTA GCT GAG TAG CGG ATA CCG-3′ (reverse primer). These changes are silent in the overlapping E2 coding sequence but introduce two translation stop codons into E4 that direct termination of the E1^E4 protein after amino acid 16. The complete genomes were sequenced to confirm that mutations, other than those introduced into E4, had not been introduced elsewhere during mutagenesis.

Cell transfections

Isolation of normal human foreskin keratinocytes from neonatal foreskin and maintenance of cells in SFM keratinocyte growth media (Invitrogen-Gibco) has been described previously (Wilson et al., 2005). Plasmids containing the wildtype and mutant E4 genomes were digested by *Eco*RI to release the HPV18 genomes, and the genomes re-circularized in the presence of T4 DNA ligase (400 U/ml, New England Biotechnologies) prior to co-transfection with a plasmid containing a neomycin resistance gene, into second-passage HFKs, using the protocols described (Wilson et al., 2005). Subsequent selection of cells in G418- and serum-containing medium (E medium) for 8 days and the culture of pooled clones on feeder layers of mitomycin C-treated J2-3T3 fibroblasts, was as described (Wilson et al., 2005).

Methylcellulose suspension

Cell differentiation was induced in monolayer cultures of untransfected and HPV18-transfected keratinocytes by transfer of cells to 1.5% methylcellulose prepared in E medium as described previously (Wilson et al., 2005).

Organotypic raft culture

Preparation of organotypic rafts cultures was as previously described (Wilson et al., 2005). Briefly, each HPV18-containing keratinocyte line was grown in organotypic raft culture by seeding onto a collagen bed containing J2-3T3 fibroblasts, and transferred onto a metal grid once a confluent state had been reached. Cell stratification was allowed to proceed for 13 days and the rafts then harvested. Cellular DNA synthesis was monitored by addition of 20 μM BrdU to the raft culture medium 12 h prior to harvest. Tissue sections were prepared from formaldehyde fixed and paraffin-embedded rafts. If rafts were labelled with BrdU, sections were immunostained with an antibody specific for BrdU.

For examination of E4 expression, the formaldehyde-fixed raft sections were treated using a low temperature method of antigen retrieval (Watson et al., 2002) and then incubated with rabbit polyclonal antibodies raised against an glutathione-*S*-transferase HPV18 E1^E4 fusion protein. Immune complexes were identified by a rabbit IgG-specific Alexa® 594 conjugate (Molecular Probes Inc) and nuclei stained with 4′, 6′-diamidino-2-phenylindole (DAPI), prior to mounting in ProFade (Molecular Probes Inc).

FISH

Fluorescence in situ hybridization (FISH) using digoxigenin labelled HPV18 genomic probe was performed on formaldehyde-fixed raft sections as previously described (Wilson et al., 2005).

Southern and Northern analysis

Protocols for DNA extraction and Southern and Northern analysis of nucleic acids isolated from HPV genome-containing keratinocyte lines have been described elsewhere (Wilson et al., 2005). Briefly, for Southern analysis, total genomic DNA was

prepared from cell cultures using the described method, or by using a DNA extraction kit (Nucleon HT, Amersham Biosciences). Samples (5 μg) were treated with *Dpn*I to digest residual input DNA and *Bgl*II. No *Bgl*II sites exist in the HPV18 genome. The digested DNAs were run on 0.8% agarose gels and DNA transferred to GeneScreen™ nylon membrane (PerkinElmer, Wellesley, MA). Complete HPV18 genomic DNA was released from the pGEMII backbone by digestion with *Eco*RI, purified and labelled with [α-^{32}P]-CTP (Amersham BioSciences). The membrane was incubated with the radiolabelled HPV18 linear probe at 42 °C overnight and following washing was exposed to autoradiograph film or band intensities quantitated by PhosphorImager analysis (Molecular Dynamics, GE Healthcare Ltd). RNA samples were extracted from cell cultures using RNA STAT-60 (ams Biotechnology, Abingdon, Oxon, UK), according to manufacturer's instructions, and Northern analysis performed using a radiolabelled HPV18 probe as described.

Western analysis

Total protein was extracted from keratinocytes suspended in methylcellulose by lysis in 9 M urea, 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.15 M β-mercaptoethanol. Equal amounts of protein were separated by SDS-polyacrylamide electrohoresis, transferred to nitrocellulose membrane and the membrane incubated with a monoclonal antibody 1D11 (Roberts et al., 2003) specific for the N-termini of E1^E4 proteins. After incubation with anti-mouse IgG specific peroxidase conjugate (Sigma Chemicals) the membrane was developed by chemiluminescence (Amersham Biosciences).

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